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Applicant(s):	Callegaro et al.	Confirmation No.:	9321
Serial No.:	09/743,333	Art Unit:	1618
Filed:	February 21, 2001	Examiner:	Blessing M. Fubara
Title:	"Biocompatible and biodegradable composition containing hyaluronic acid and the derivatives thereof for the treatment of the digestive tract of the ulcers"		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 C.F.R. § 1.132

I, Anna Zanellato, being duly sworn depose and say that:

1. I am an Italian citizen residing at: Bovolenta (PD)

2. I am familiar with the English language.

3. I graduated in: BIOLOGY at the University of Padua in the academic year: 1987

4. I am author of 19 Scientific publications.

5. Previous job experiences: From 1987 to 1990 I had worked at the University Department of General Pathology as a researcher, where I had been involved in a study pertaining to smooth muscles cells cultures and in particular to the mechanism of atherosclerosis.

6. Actual job: Since 1990 I have been working at FIDIA FARMACEUTICI S.p.A. in the field of research, involving:

- the analysis of action mechanism of trophic factors,
- studies, utilizing neuronal cultures to select new chemical molecules pharmacologically active to prevent different types of neuronal pathologies,
- other studies concerning bovine, rabbit, human, articular chondrocytes cultures on the biomaterials comprising and/or consisting of hyaluronic acid derivatives.

The following tests had been carried out under my own responsibility.

Experimental section

Enterocytes ($CaCO_2$ cell line that differentiates into enterocytes typical of the mature intestinal epithelium) were seeded onto supports made of the total benzyl ester of hyaluronic acid in the form of :

- a bidimensional continuous membrane, as shown in Figure A below,
- a bidimensional perforated membrane (Laserskin®), and
- a non-woven 3-D matrix, as shown in Figure B below,

in order to test their biocompatibility, and their morphological and biochemical responses were observed.

Figure A shows the 2-D continuous membrane as raw material for the purposes of the current invention, whereas the perforated membrane is the same 2-D continuous membrane where regularly spaced openings have been made.
Figure B shows a 3-D non-woven matrix made of Hyaff®, which is the trademark referring to total benzyl ester of hyaluronic acid. Also the above continuous and perforated membrane are made of Hyaff®, in order to have the comparison among supports as significant as possible.

The cells $CaCO_2$ were used at passage 98. The cells were seeded at a density of about $9\times10^3/cm^2$ in DMEM 4.5g of glucose/L containing 20% FBS penicillin/streptomycin, fungizone and non-essential amino acids (1%) in a humidified atmosphere with 95% CO_2. The culture medium was changed every 48 hours.
Other enterocytes were seeded on the following different supports:

- Petri dishes,
- Transwell wells with polycarbonate membranes, and
- Polyurethane membranes (Chronoflex ™).

On the 3rd, 15th, 20th and 40th days of culture, the cells were prepared for assessment of the total proteins and the activity of alkaline phosphates (ALP) according to the following method:

ALP activity: the cells were harvested by scraping in a lysis buffer 2mM Tris-HCl 50 mM mannitol pH 7.2 (1 ml final volume) (with the exception of those seeded on Hyaff 3D) and sonicated in ice. ALP activity of the cellular lysates was determined by spectrophotometry by hydrolysis of the p-nitrophenylphosphate using a Boehringer kit. The total proteins were determined by Lowry's method. The activity present in the cells grown on a scaffold in the form of a non-woven fabric was determined in lysates obtained by sonicating the membrane containing the cells in toto. Values of APL activity are expressed as milliunits per milligram protein mU/mg.

The biochemical differentiation was assessed on the basis of the increase of ALP activity that is a known Enterocyte Differentiation Marker.

Results

In Figure C, the results of ALP activity obtained for $CaCO_2$ cell line grown on

- Petri dishes
- Transwell wells with polycarbonate membranes
- Polyurethane membranes (Chronoflex™)
- a bidimensional continuous membrane, see Figure A
- a bidimensional perforated membrane (Laserskin®);
- a 3-D non-woven Hyaff® matrix, see Figure B.

are shown, where the APL activity is reported after 3, 15, 20 and 40 days for all the above supports respectively.

It should be easily noticed that **the bidimensional continuous and perforated membranes show an unexpectedly great improvement in APL activity with respect to the 3-D non-woven matrix, even when the same raw material is used**. Therefore, contrary to the Examiner's assertion, **the enterocytes do not grow and do not differentiate in 3-D scaffolds in a surprisingly way as in 2-D membranes**. The growing enterocytes on the continuous bidimensional membrane of the invention are shown in Figure D below.

However, the Experiment show that not all the 2-D supports can be suitable for enterocytes. In fact, **the bidimensional continuous and perforated membranes show an unexpectedly great improvement in APL activity also with respect to the Petri dishes, the transwell wells with polycarbonate membranes and the polyurethane membranes (Chronoflex™).**

This confirms with no doubt that morphological differentiation is not a natural development of intestinal seeded cells, as simplicistically concluded by the Examiner, since it has been demonstrated that **only bidimensional continuous and perforated membranes consisting essentially of at least one hyaluronic acid or a derivative thereof can allow to achieve morphologically differentiated enterocytes as confirmed by the presence of microvilli, in order to obtain a biological material suitably *configured* for the treatment of ulcers, lesions and diverticula of the digestive and gastrointestinal apparatus**. The presence of microvilli has been already noticed in the Example given in the Application as filed, where reference is made to Figure 3 showing "*marked differentiation due to the appearance of numerous microvilli on their surfaces*".

It should be also noted that **the APL activity in case of the bidimensional *continuous* membrane is better than the APL activity in case of the bidimensional *perforated* membrane.** Therefore, this is a further surprising and definitely unexpected result achieved by the invention, particularly in view of all the prior art teachings!

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: March 30, 2009

Anna Zanellato

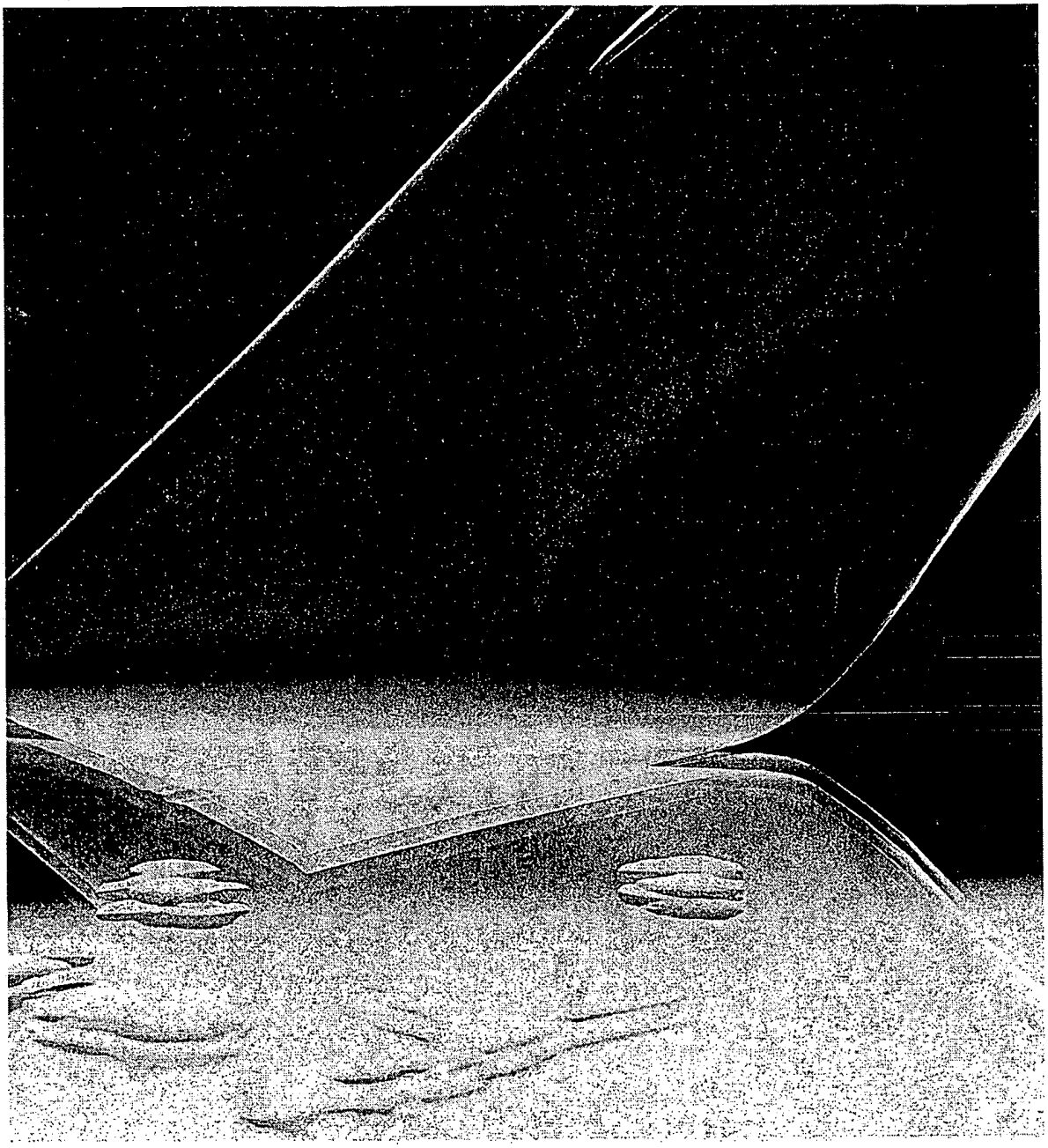
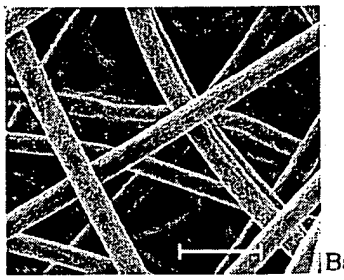


Figure A



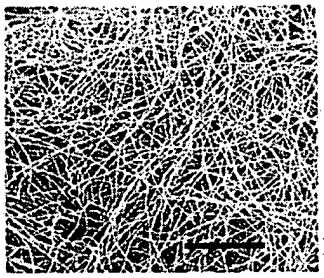


Figure B

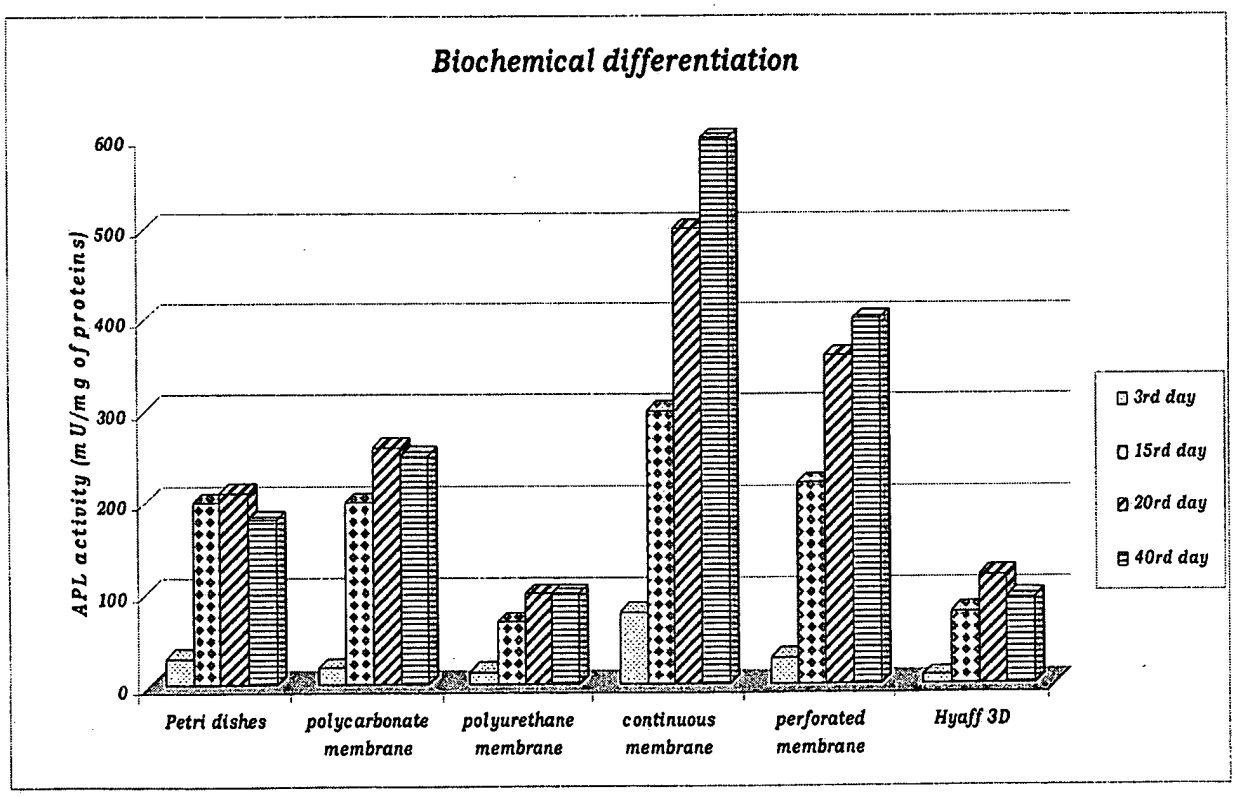


Figure C

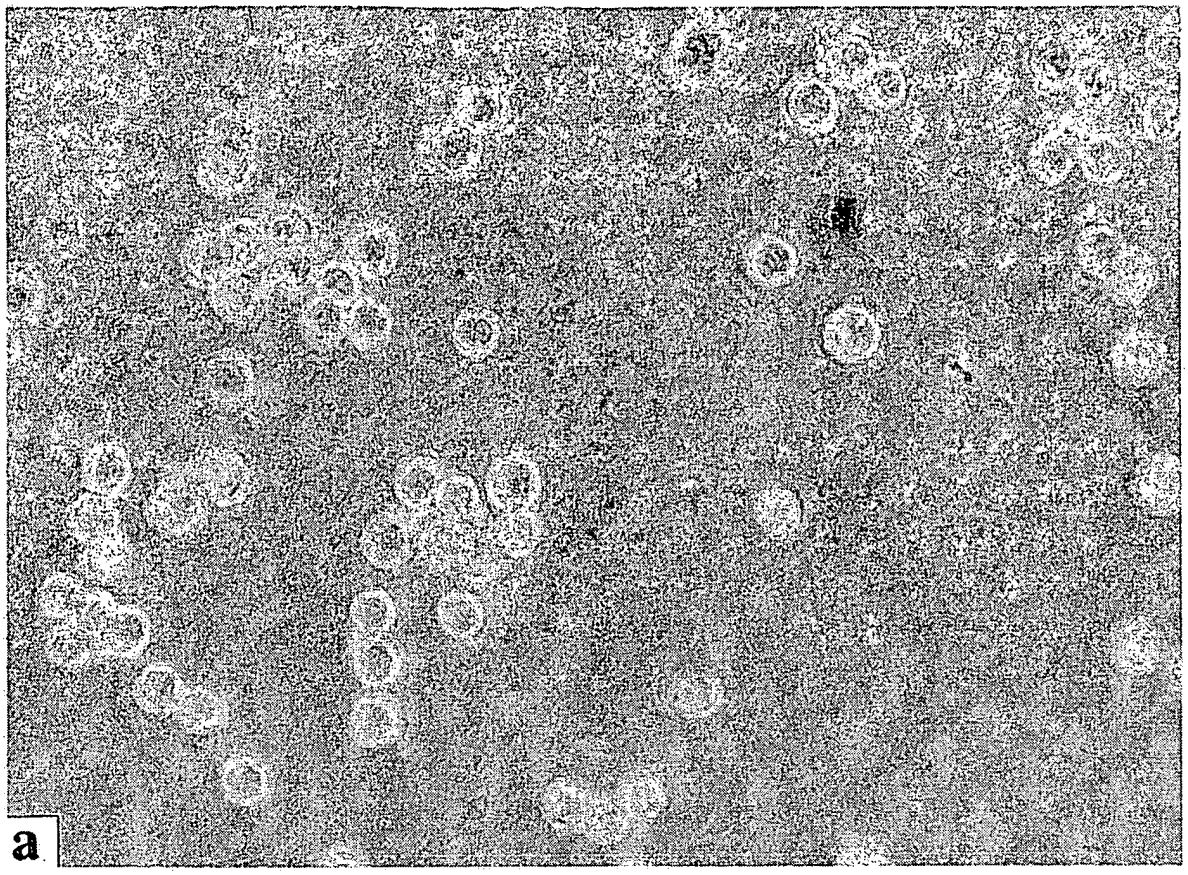


Figure D